



Biotechnology & Biotechnological Equipment

ISSN: 1310-2818 (Print) 1314-3530 (Online) Journal homepage: https://www.tandfonline.com/loi/tbeq20

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To cite this article: Michelle Arredondo, Margarita Stoytcheva, Israel Morales-Reyes & Nikola Batina (2018) AFM and MFM techniques for enzyme activity imaging and quantification, Biotechnology & Biotechnological Equipment, 32:4, 1065-1074, DOI: 10.1080/13102818.2018.1470904

To link to this article: https://doi.org/10.1080/13102818.2018.1470904

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Published online: 07 May 2018.

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ARTICLE; BIOTECHNOLOGICAL EQUIPMENT

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AFM and MFM techniques for enzyme activity imaging and quantification

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ABSTRACT

In this work, a new approach for enzyme activity monitoring is suggested. It is based on the realtime imaging, by atomic force microscopy (AFM) and magnetic force microscopy (MFM), of the degradation of a nanoparticles-loaded enzyme responsive layer, namely Fe_3O_4 -nanoparticlesloaded gelatin. The roughness analysis allowed the quantification of the trypsin-induced ferrogel degradation and the correlation of the AFM and MFM data obtained. ARTICLE HISTORY Received 12 January 2018 Accepted 26 April 2018

KEYWORDS Atomic force microscopy; magnetic force microscopy; trypsin activity; ferrogel

Introduction

Nowadays, the atomic force microscopy, developed in the mid-1980s [1], is recognized as one of the most important tools for imaging, measuring, analyzing and manipulating matter at the nanometer scale. The basic principles of the technique and its multiple applications in physical, chemical, material and life sciences have been extensively reviewed and reported in the literature [2]. It is noteworthy to highlight that the ability of the atomic force microscopy to be used in liquid media and to image non-conductive surfaces makes it particularly suitable for biological samples characterisation. Biological applications include: (i) direct imaging of molecules, cells, tissues and biomaterials and (ii) molecular interactions investigation, such as DNA replication, protein synantigen-antibody, and receptor-ligand thesis, interactions, among others [3–7]. The technique has also been applied for enzyme-substrate interactions studies and characterization of immobilized enzyme molecules on biosensor surfaces [8–11], but only a few papers comment on the atomic force microscopy application to enzyme activity exploration. Radmacher et al. [12] report direct observations of enzyme activity based on the detection of the height fluctuations of the cantilever. Single-binding events between acetylcholinesterase and its substrate acetylcholine have been shown by recording force spectra [13,14]. Indirect mapping of enzyme activity was demonstrated by imaging phospholipase and the time course of lipid bilayer degradation [15-18]. Other enzyme activity and kinetics studies are based on the detection of the molecular byproducts during enzymatic conversion [19,20]. Recent studies demonstrate that, although AFM enables several research options [21,22], its capability for enzyme activity imaging remains not completely exploited.

In this work, a new approach for enzyme activity monitoring is suggested. It is based on the real-time imaging, by atomic force microscopy (AFM) and magnetic force microscopy (MFM), of the degradation of a nanoparticles-loaded enzyme responsive layer, namely Fe₃O₄nanoparticles-loaded gelatin. During the enzymatic gelatin degradation by trypsin as an example, the nanoparticles also leave the sensitive layer together with the substrate degradation products, as previously confirmed by quartz crystal microbalance (QCM)-based measurements [23,24].

Materials and methods

Reagents

Gelatin from porcine skin (Type A, G2500) and trypsin from bovine pancreas (T9201, \geq 7500 BAEE units/mg solid) were purchased from Sigma. All the other chemicals were of analytical reagent grade and were used without further purification. Enzyme activity was verified applying the standard spectrophotometric assay protocol of Sigma–Aldrich [25]. Data obtained under different storage conditions were consistent with those defined by the manufacturer.

Fe₃O₄-nanoparticles-loaded gelatin hydrosol was prepared as described by Gordon et al. [26]. In brief, 240 μ L FeCl₂.4H₂O solution (10 mmol/5 mL 0.01 N HCl) were

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added to 80 mL aqueous solution containing 240 mg porcine gelatin, followed by the addition of 86 μ L NaNO₃ solution (6 mmol/5 mL H₂O). After a reaction time of 10 min, the pH was raised to 9.5 by adding an aqueous solution of NaOH (1 N). This procedure was repeated four times more. Excess reagents were removed by extensive dialysis against water, which also neutralised the nanoparticle dispersion. The obtained hydrosol was kept at 4 °C and was stable for several months. Ferrogels formation was achieved as described below.

Atomic force microscopy (AFM) and magnetic force microscopy (MFM)

Ferrogel surface imaging was performed by using a commercial Nanoscope III AFM (Digital Instruments, Santa Barbara, California, USA). The experiments were carried out in air at ambient conditions, applying the imaging technique 'tapping mode' with a standard geometry probe model TESP (Si) in the frequency range of 325– 382 kHz (Veecoprobes). All images were taken in flatten mode and a scan size of 5 μ m at a relatively low scan rate (ca. 1 Hz) to avoid sample damage.

MFM measurements were performed in lift mode. Soft tapping conditions were used to avoid particle displacements, surfactant indentation or height artifacts [27]. The tip magnetisation was directed perpendicularly to the sample surface. The magnetic signal was recorded using the phase-detection technique. Magnetic tape from Digital Instrument was used as a control. To improve imaging, the AFM and MFM scans were decoupled and tuned separately. The drive amplitude of 2 V combined with the lift height of 100 nm resulted in an improved signal-to-noise ratio. Hence, despite the reduction of the intensity of the signal, the magnetic features were better resolved as described by Kebe et al. [28].

Image analysis

Magnetization versus particles density per unit area was evaluated using commercial software SPIPTM (Scanning Probe Image Processor) version 6.2.4 from Image Metrology A/S, HorshØlm, Denmark, in order to quantify the total number of particles. The particle and pore analysis module was selected to compare topography-(AFM) and magnetic-(MFM) with watershed-packed features. For features detection, the image was coloured randomly, according to the height ratio. The smooth shape contours offered discrimination of fixed features for particle agglomerations larger than 100 nm. Advanced settings, e.g. detail level, finest filtering and slope noise reduction, were set up to achieve coverage of about 97% or more.

Sample preparation

The Fe₃O₄-nanoparticles-loaded gelatin hydrosol (8 μ L) was dropped onto the surface of a QCM gold-coated crystal (AT-cut/8 MHz, 13.7 mm crystal diameter and 5.11 mm diameter of the gold disk). The Fe₃O₄-gelatin hydrosol was dried for 1 h at ambient temperature to obtain a ferrogel, which was used as a trypsin substrate and sample for AFM and MFM studies. The QCM-sensors for enzyme activity determination, described in our previous works were constructed applying a similar protocol [23,24]. The sample assembling scheme is presented in Figure 1.

Results and discussion

AFM imaging of the ferrogel degradation

The principal impetus for the characterization of the ferrogel surface was to demonstrate the possible correlation between surface degradation and trypsin activity. Above all, the topology change sequence presented in Figure 2 demonstrated that the trypsininduced ferrogel degradation is time dependent. The deposited ferrogel layer before its exposure to the trypsin action is uniform (Figure 2(a)). A complete surface coverage with homogeneously distributed nanoparticles, as well as the formation of a small number of agglomerations was observed. Ferrogel exposure to trypsin for 5 min provoked drastic modifications (Figure 2(b)). The uniformity of the ferrogel layer was altered and its roughness was markedly increased. The topological changes were attributed to the strong interaction between the enzyme and its substrate and the rapid dissolution of the obtained products. Such a degradation model was suggested in our previous works to explain the increase of the frequency response of the QCM-based sensors for enzyme activity evaluation [23,24]. Longer exposure time (30 min) resulted in layer flattening, followed by the almost complete ferrogel degradation within 60 min of exposure time, and roughness increase, as illustrated by Figure 2(c,d). The comparable topographies of the ferrogel layer after a 60 min exposure time to the trypsin action (Figure 2(d)) and the topography of the unmodified gold-coated quartz crystal (Figure 2(e)) confirmed that the layer degradation process was achieved. These results clearly demonstrate that AFM-imaging could be successfully applied for monitoring the progress of the degradation of the enzyme responsive layer deposited onto the QCM surface and the enzyme activity could be correlated with the layer topography changes.



Figure 1. Sample assembling scheme.



Figure 2. Trypsin-induced ferrogel degradation monitoring: 0 min (a), 5 min (b), 30 min (c) and 60 min (d) exposure time; and unmodified gold-coated quartz crystal (e). Note: Trypsin concentration: 0.1 μ g/mL.



Figure 3. Topography (a) and magnetic phase (b) image of the QCM untreated surface. Note: Images scale 5 μ m.

MFM imaging of the ferrogel degradation

MFM is an advanced method to study magnetic surfaces, including non-conductive samples. Magnetic long-range interactions are measured by the exclusion of the conduction electrons [29].

In the present work, the selected area in all experiments was previously delimited and indicated. The obtained AFM and MFM images of the untreated and unmodified with a ferrogel QCM surface are shown in Figure 3. The dark and light areas on the AFM image correspond to the depths and heights of the surface, respectively. The MFM image did not display a significant colour change, thus confirming the absence of magnetic behaviour, as expected for the surface without any treatment. The additional AFM measurements demonstrated that the modification of the QCM surface by a ferrogel results in the formation of a smooth and uniform film (Figure 4(a)), as commented in the previous section. The MFM image (Figure 4(b)) revealed the appearance of several deeps in dark colour, which corroborates with the presence of magnetism, due to the Fe_3O_4 nanoparticles incorporation.

Further experiments visualize the progress of the ferrogel degradation process with time during ferrogel exposure to the trypsin action by MFM imaging (Figure 5). The appearance of increasing dark areas indicates the enhancements of the magnetic properties with time, which was attributed to the progressive gelatin digestion and appearance of uncoated Fe_3O_4 nanoparticles.



Figure 4. Topography (a) and magnetic phase (b) image of the ferrogel film deposited onto the QCM surface. Note: Images scale 350 nm.



Figure 5. Magnetic phase image of ferrogel film exposed to trypsin action: 5 min (a), 30 min (b), 60 min (c) of exposure time. Note: Images scale 5 μ m.

AFM and MFM images correlation

The roughness analysis allows the quantification of the trypsin-induced ferrogel degradation and the correlation of the AFM and MFM data obtained. For this purpose, the values of the root mean square average of the profile heights over the evaluation length (RMS) were assessed. RMS values were plotted as a function of the time of exposure of the ferrogel to the trypsin action, as shown in Figure 6.

As reported before, the heavy nanoparticles and products of the gel enzymatic degradation leave the substrate layer, promoting a great decrease of the QCM crystal mass. The calculated RMS values obtained from the AFM measurements confirm the above-reported findings. In addition, the measurements in MFM mode showed a similar degradation tendency and an increase of the magnetic force gradient by prolonged enzyme activity. It results from the surface exposure of uncoated Fe₃O₄ magnetic nanoparticles on the surface top layer as a consequence of the gelatin digestion process (Figure 5). Hence, the combination of AFM and MFM techniques offers complementary results to the study by conventional QCM analysis, because of the monitoring of the progress of the superficial changes during enzyme activity.



Figure 6. Quantification of the trypsin-induced ferrogel degradation.



Figure 7. Particle analysis: QCM surface (a); ferrogel surface before its exposure to trypsin action (b) and after 5 min (c), 30 min (d) or 60 min (e) of exposure to trypsin action. Note: Analysed area $5 \times 5 \mu$ m.



d



Figure 7. (Continued)



Figure 7. (Continued)

Particle quantification

Surface image processing and data analysis were performed to quantify the nanoparticles present on the surface of the studied sample. The obtained AFM top view, MFM phase top view, and MFM top view images are shown in Figure 7. The first one of each two rows presents the non-processed images and the second one presents the images processed by particle analysis.

The number of particles determined by analyzing the AFM top view and the MFM phase top view images obtained in identical conditions was practically constant. This result is due to the specific morphology information provided by the methods applied. For comparison, an increased number of particles were found by analyzing the MFM top view images, which allowed confirming the enzymatic degradation of the gelatin layer leading to the appearance of uncoated magnetic nanoparticles. The obtained data were summarized and graphically presented in Figure 8.

The decrease in the number of the Fe_3O_4 nanoparticles on the sample surface with the time of exposure to the trypsin action completely corroborates with the results obtained by QCM analysis [23]. The release of nanoparticles during the enzymatic substrate degradation by trypsin, i.e. the reduction of their number onto the QCM surface caused a QCM frequency increase. It was clearly noticed that the film degradation and magnetic particles release occurs primary during the first 5–7 min. It is in perfect agreement with the changes in the film surface roughness presented above. Such a combination of AFM and MFM techniques allows monitoring of the film surface properties at the nanometric level and offers a new insight to the study of the formation and wearing-decomposition of modified-functionalised surfaces, including highly complex multi-layered nanodrugs.

The obtained results are in very good agreement with a previous study, where the QCM frequency change for the same samples was measured [23]. It was found that the QCM frequency decreases significantly by higher enzyme activity, due to the very rapid film degradation. By such comparison, it could be concluded that AFM and MFM are effective tools for enzyme activity monitoring and nanostructured surfaces characterization. They offer valuable data for complete interpretation of the QCM measurements. In addition, we suggest using the AFM and MFM techniques for detailed analysis of complex multi-layered modified surfaces or assemblies, like nano-drugs.



Figure 8. Total number of particles change with ferrogel time of exposure to trypsin action.

Conclusions

AFM and MFM were applied for imaging and quantification of the trypsin-induced ferrogel degradation. It was demonstrated that the topological modifications provoked by the enzyme action could be correlated with the magnetic behaviour of the Fe₃O₄ nanoparticles. All findings show that the magnetic nanocomposite hydrogel film degradation is very fast (5–7 min) and that the magnetic particles leave the film surface due to gelatin film decomposition. MFM analysis allows us to assess the magnetic particle distribution after every step of degradation, at nanometric scale. The AFM and MFM techniques could be used for detailed analysis of nano-drugs.

Acknowledgements

This study was supported by the CONACyT Ph.D. scholarship support 256887 and the Postgraduate Programs of the Engineering Institute of the UABC and of the Chemistry and Basic Science and Engineering Division of UAM-Iztapalapa.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

- [1] Binnig G, Quate CF, Gerber CH. Atomic force microscopy and drug induced degradation. Phys Rev Lett. 1986;56:930–933.
- [2] Eaton P, West P. Atomic force microscopy. Oxford (UK): Oxford University Press; 2010. p. 256.

- [3] Kasas S, Thomson NH, Smith BL, et al. Biological applications of the AFM: from single molecules to organs. Int J Imaging SystTechnol. 1997;8:151–161.
- [4] Willemsen OH, Snel MM, Cambi A, et al. Biomolecular interactions measured by atomic force microscopy. Biophys J. 2000;79(6):3267–3281.
- [5] Jalili N, Laxminarayana K. A review of atomic force microscopy imaging systems: application to molecular metrology and biological sciences. Mechatronics. 2004;14 (8):907–945.
- [6] Hilal N, Bowen WR, Alkhatib L, et al. A review of atomic force microscopy applied to cell interactions with membranes. Chem Eng Res Des. 2006;84(4):282–292.
- [7] Allison DP, Mortensen NP, Sullivan CJ, et al. Atomic force microscopy of biological samples. WIREs Nanomed Nanobiotechnol. 2010;2:618–634.
- [8] Zhang P, Tan W. Atomic force microscopy for the characterization of immobilized enzyme molecules on biosensor surfaces. Fresenius J Anal Chem. 2001;369(3–4):302–307.
- [9] Marcuello C, de Miguel R, Gómez-Moreno C, et al. An efficient method for enzyme immobilization evidenced by atomic force microscopy. Protein Eng Des Sel. 2012;25 (11):715–723.
- [10] Saal K, Sammelselg V, Lõhmus A, et al. Characterization of glucose oxidase immobilization onto mica carrier by atomic force microscopy and kinetic studies. Biomol Eng. 2002;19(2–6):195–199.
- [11] Mohamad NR, Marzuki NHC, Buang NA, et al. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. Biotechnol Biotechnol Equip. 2015;29(2):205–220.
- [12] Radmacher M, Fritz M, Hansma H, et al. Direct observation of enzyme activity with the atomic force microscope. Science. 1994;265(5178):1577–1579.
- [13] Yingge Z, Delu Z, Chunli B, et al. Force spectroscopy between acetylcholinesterase molecule and its natural

substrate to study the effects of inhibitors and reactivators on enzyme activity. Life Sci. 1999;65(21):PL253– PL260.

- [14] Yingge Z, Chunli B, Chen W, et al. Force spectroscopy between acetylcholine and single acetylcholinesterase molecules and the effects of inhibitors and reactivators studied by atomic force microscopy. J Pharmacol Exp Ther. 2001;297(2):798–803.
- [15] Nielsen LK, Risbo J, Callisen TH, et al. Lag-burst kinetics in phospholipase A2 hydrolysis of DPPC bilayers visualized by atomic force microscopy. Biochim Biophys Acta-Biomembranes. 1999;1420(1–2):266–271.
- [16] Grandbois M, Clausen-Schaumann H, Gaub H. Atomic force microscope imaging of phospholipid bilayer degradation by phospholipase A2. Biophys J. 1998;74(5):2398– 2404.
- [17] Balashev K, Jensen TR, Kjaer K, et al. Novel methods for studying lipids and lipases and their mutual interaction at interfaces. Part I. Atomic force microscopy. Biochimie. 2001;83(5):387–397.
- [18] Wu H, Yu Le, Tong Y, et al. Enzyme-catalyzed hydrolysis of the supported phospholipid bilayers studied by atomic force microscopy. Biochim Biophys Acta-Biomembranes. 2013;1828(2):642–651.
- [19] Kranz C, Kueng A, Lugstein A, et al. Mapping of enzyme activity by detection of enzymatic products during AFM imaging with integrated SECM–AFM probes. Ultramicroscopy. 2004;100(3–4):127–134.
- [20] Kueng A, Kranz C, Lugstein A, et al. Integrated AFM–SECM in tapping mode: simultaneous topographical and

electrochemical imaging of enzyme activity. Angewandte Chemie Int. 2003;42:3238–3240.

- [21] Maver U, Velnar T, Gaberšček M, et al. Recent progressive use of atomic force microscopy in biomedical applications. Trend Anal Chem. 2016;80:96–111.
- [22] Variola F. Atomic force microscopy in biomaterials surface science. Phys Chem Chem Phys. 2015;17:2950–2959.
- [23] Stoytcheva M, Zlatev R, Cosnier S, et al. High sensitive trypsin activity evaluation applying a nanostructured QCM-sensor. Biosens Bioelectron. 2013;41:862–866.
- [24] Stoytcheva M, Zlatev R, Velkova Z, et al. Nanoparticle modified QCM-based sensor for lipase activity determination. Analyt Method. 2013;5:3811–3815.
- [25] Bergmeyer HU, Gawehn K, Grassl M. Methods of enzymatic analysis. 2nd ed. Vol. I. New York: Academic Press; 1974. p. 515–516.
- [26] Gordon T, Perlstein B, Houbara O, et al. Synthesis and characterization of zinc/iron oxide composite nanoparticles and their antibacterial properties. Colloids Surfaces A Physicochem Eng Aspects. 2011;374(1–3):1–8.
- [27] Ebenstein Y, Nahum E, Banin U. Tapping mode atomic force microscopy for nanoparticle sizing: tip-sample interaction effects. Nano Lett. 2002;2(9):945–950.
- [28] Kebe Th, Carl A. Calibration of magnetic force microscopy tips by using nanoscale current-carrying parallel wires. J Appl Physics. 2004;95:775–792.
- [29] Puentes VF, Goroztiza P, Aruguete DM, et al. Collective behaviour in two-dimensional cobalt nanoparticle assemblies observed by magnetic force microscopy. Nat Materials. 2004;3:263–268.